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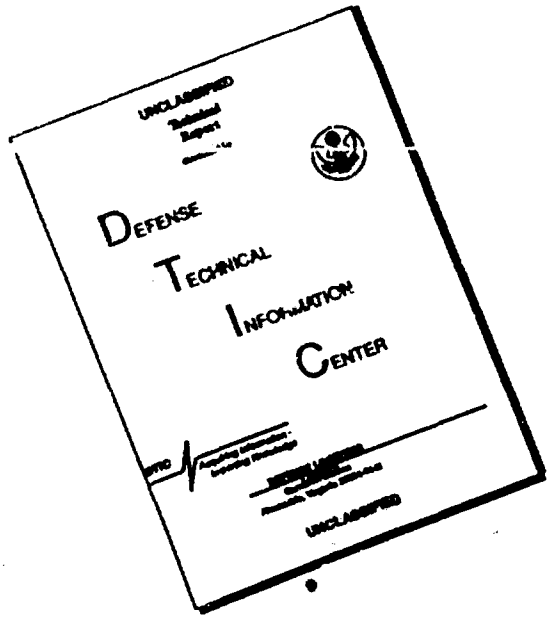

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THE POSSIBILITY OF THE EXPERIMENTAL TRANSFORMATION OF VARIOLA MAJOR VIRUS INTO VACCINIA VIRUS

[Following is thr translation of an article by V. D. Solovyev, Yu. N. Mastyukova, N. V. Yaroslavskaya and N. T. Saraťeva, Virology Faculty of the Central Institute for the Advancement of Doctors and the Moscow Scientific-Research Institute of Epidemiology and Microbiology, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology) No 3, 1965, pages 307--315. It was submitted on 28 Jul 64. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

The problem concerning the possibility of attenuating and transforming the variola major virus into the vaccinia virus experimentally is of interest not only in a theoretical aspect but is also of practical importance, since its solution is connected with attempts at obtaining vaccine strains from freshly isolated strains of the variola major virus. This problem has a history of many years, has been subjected to numerous discussions in the press, and has had numerous experimental revisions. The literary data available in this respect was pointed out by us earlier /4/. Here we will only point out that in recent years the feasibility of the experimental transformation of the variola major virus into the vaccinia virus has become doubtful, and the positive results obtained by a number of authors by passaging the smallpox virus through animals are explained by the fact that a mixed population of the variola major virus and the vaccinia virus were used as the initial material /8/. Since in contrast to the smallpox virus the vaccinia virus possesses a wide spectrum of pathogenicity and multiplies intensively in various cellular systems, it is suggested that the multiplication of vaccinia virus, which is latently present in the inoculated material, was mistaken for the transformation of the smallpox virus. Several possibilities are pointed out for the contamination of the smallpox virus with vaccinia virus: Vaccination of persons during the smallpox incubation period; in laboratories preparing the vaccine; following the infection of animals, naturally infected with the vaccinia virus, with the variola major virus.

Taking into consideration that the majority of investigators observed changes in the properties of the variola major virus with its transformation into vaccinia virus following the inoculation of variolar material under the hide of cows and bull calves, we decided to carry out a study of the properties of the variola major virus during the process of prolonged cultivation in a cell culture from the skin of a bovine embryo and compare them with the properties of the same virus, cultivated in a cell monolayer of a human embryo, and the proberties of vaccinia virus strains and genuine cowpox.

Materials and Methods

The study was carried out with two strains of the variola major virus, isolated from the scabs of patients: One - by the infection of developing chick embryos, and the other - by the infection of a cell culture from the lungs of a human embryo.*

For passaging the variola major virus we used 4--5-day old cell monolayers from the skin of a bovine and human embryo, prepared according to the method which was described earlier /4/. As the material for infection during the process of passaging the virus we used the cultural fluid, collected from the cell cultures during the period of onset of complete specific degeneration. The cultural fluid was placed in test tubes with cultures in a volume of 0.1 ml. After every 5--7 subsequent passages in cell cultures we studied the properties of the smallpox virus by means of titration in the chorio-allantois of chick embryos, in a culture of HEp-1 cells and cells of chick embryos. The cultures of cells were prepared according to the method described earlier /4/, and were infected on the 2--3rd day of cell growth. In doses of 0.1 ml, each 10-fold dilution of virus suspensions was placed in 4 test tubes with cell cultures and on the chorio-allantois of 4--12-day old chick embryos. The results were considered in the chick embryos on the 5th day of incubation at 37°, and in the cell cultures daily for the course of 9 days of incubation at the same temperature.

The properties of the variola major virus were compared with the properties of 3 strains of dermovaccine virus (Gamaleya Institute of Epidemiology and Microbiology, AMN USSR, Odessa Institute of Vaccines and Sera, Minsk Institute of Epidemiology, Microbiology and Hygiene), one strain of ovovaccinia, which had made 531 passages through developing chick embryos, and one strain of the genuine cowpox virus, isolated in chick embryos from animals during the period of an outbreak of this disease. The properties of the stated viruses were studied by means of titration on rabbit skin, in developing chick embryos, cultures of HEp-1 cells and the fibroblasts of chick embryos in the hemagglutination reaction (HA).

Ten-fold dilutions in a volume of 0.1 ml were administered subcutaneously for derterming the infectious activity of viral suspensions on rabbits. The reaction was evaluated on the 4--5th day following infection. This was done by calculating the end dilution of viral suspension causing the development of an infiltrate on the skin of the rabbits and measuring the width of the infiltrates and computing the sums.

The HA was set up with a 1% suspension of chicken erythrocytes at room temperature /4/.

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*The strains were isolated during a smallpox outbreak in Moscow from the end of 1960 to the beginning of 1961.

Results

The investigations showed (see table) that the variola major virus can be differentiated from the cowpox and vaccinia virus based on the nature of the morphological changes caused on the choric-allantois of developing chick embryos and in a culture of HEp-1 cells, and also based on the absence of a cytopathological effect in a culture of chick embryo fibroblasts. Thus, on the chorioallantois of chick embryos both strains of the variola major virus caused the development of minute compact arched lesions, surrounded by a hyaline peripheral zone (figure 1, a). The cowpox virus caused lesions of various size, as a rule ring-shaped, with a hyaline center and thickened periphery; often the lesions had a mixed nature, especially at the site of inoculation of the virus (figure 1, b). In general the lesions caused by ovovaccinia virus were large, most often of a circular form, diffuse, with a hyaline periphery and a more dense, often ulcerous center (figure 1, c); the lesions caused by the 3 strains of dermovaccine had a similar nature. The variola major virus did not display a cytopathological effect in a culture of fibroblasts of trypsinized chick embryos; the cowpox and vaccinia viruses under the same conditions produced an expressed cytopathological effect.

It is interesting to note that analogous data were obtained by other investigators, thus, Mika and Pirsch /11/, while studying the formation of plaques by a number of smallpox viruses in a cell culture of chick embryo under an agar layer at 36°, observed that the viruses of vaccinia (3 strains), cowpox (white and red variants), rabbitpox and monkeypox formed plaques; variola (13 strains) and alastrim did not cause the formation of plaques. The authors suggest using the inability of the variola virus to produce plaques in a culture of chick embryo fibroblasts along with the inability to be sustained in passages in rabbit epidermis and the characteristic morphology of the pocks on the chorio-allantois of developing chick embryos as criteria for differentiating it from the vaccinia virus.

In our investigations in a culture of HEp-1 cells the variola major virus formed very apparent, at a small magnification of the microscope, sharply outlined foci of cellular proliferation, made up of several layers of cells (figure 2, a). When a concentrated suspension of variola virus was placed in the culture the stated focal lesions were especially well apparent in the early stage of infection -- in 24--30 hours following infection; generalized necrotic changes developed subsequently. In cultures infected with small concentrations of virus the foci of cellular proliferation appeared later -- in 72--96 hours following infection. Along with the progressing of the infection the cells of such foci gradually degenerated and dropped off of the slide. There were comparatively few giant cells in the HEp-1 cell cultures, infected with variola virus, and they were small in size. We never observed a similar nature of focal cellular proliferation in cultures infected with the cowpox and vaccinia viruses.

The cowpox virus and a virus strain of ovovaccinia caused a similar cytopathological effect in the HEp-1 cell culture -- the formation of multinuclear vacuolized symplasts (figure 2, b, c). This type of cellular degeneration differed from the morphological changes, caused in the same cultures by 3 strains of dermovaccine virus. These changes were characterized by a disruption of cellular bonds, the grouping of cells, formation of cellular aggregates with the symptoms of phagocytosis, and the breaking up of the entire culture into sectors of grouped cells (figure 2, d). Vacuolized giant cells were encountered comparatively rarely.

The stated similarity in the cytopathological effect of the cowpox and ovovaccinia viruses could have been caused by the community of their origin, since the ovovaccinia strain is a derivative of the cowpox virus, which during the period of a few dozen years was cultivated only in the skin of a bull calf. It is not excluded that the cultivation of ovovaccinia and cowpox viruses in chick embryos could have exerted an influence on the nature of their cytopathological effect in cell cultures. The origin of the 3 strains of dermovaccine was not known to us, but they were passaged on the skin of other animals in addition to bull calf: Asses (strain from the N. F. Gamaleya Institute of Epidemiology and Microbiology), sheep and rabbits (strain from the Odessa Institute of Vaccines and Sera).

In connection with this we came to the conclusion that for the differentiation of variola virus the most important trait is the nature of the cytopathological effect caused by it in transplanted cell cultures. According to the observations of other investigators, this virus causes analogous morphological changes in HeLa and Fl cell monolayers [10, 12, 14].

The two other differential features are less valuable for the following reason. The lesions, caused by the variola virus on the chorioallantois of developing chick embryos, are sharply different from the lesions caused by the cowpox and vaccinia viruses only in the event that the infection is nonlethal for embryos, and are not distinguished with reliability in dead embryos. Based on literary data /13/, the cytopathological effect in a culture of chick embryo fibroblasts may not be apparent also during infection with small concentrations of vaccinia virus, which is due to the intense porliferation of the cells of the chick embryo. On the other hand, there are indications that the variola virus, having undergone a number of passages in cell cultures or in developing chick embryos, is capable of exerting a cytopathological effect on cultures of chick fibroblasts [2, 5]. Apparently this is connected not only with a change of conditions for virus cultivation, but with the increased concentration of viral particles in the inoculate. To a certain degree the observations by Higashi and Ichimiya /9/ may be a confirmation of this. Having infected a culture of HeLa cells with the variola virus, adapted to developing chick embryos, and having carried out successive passages, the authors observed cytopathological changes for the first time only after 5--6 passages. The cytopathological effect of the virus was increased considerably following 15--16 passages. At the same time a noticeable increase was noted in infecting ability: The titer of the virus from the 6th to the 20th passage was raised by 2.25 lg.

We studied the properties of the 2 strains of variola virus in a cell culture from the skin of bovine and human embryo over the extent of 50 successive passages. The results of the investigations showed that both during primary infection and during the process of successive passages the variola virus multiplied more intensively in the cell culture from the skin of a human embryo than in the cell culture from the skin of a bovine embryo. This was manifested in an earlier onset of the cytopathological effect and the complete cellular degeneration, and also in higher titers of infecting ability. For example, in cell cultures from the skin of a human embryo the cytopathological effect was displayed for the first time in 24--48 hours and ended with the complete breaking up of the culture in 6--7 days following infection; the titer of the infecting ability of the cultural fluid, collected during the period of the onset of complete cell degeneration, equaled the titers on chick embryos and in HEp-1 cell cultures: 10^{-4}--10^{-5} TCD_{50}. The cytopathological effect in cell cultures of bovine embryo skin were revealed for the first time in 72--96 hours, and the complete break up of the culture set in on the 9--10th day following infection; the titer of the infecting ability of the cultural fluid fluctuated from 10^{-3} up to 10^{-4} TCD_{50}. No other differences were observed in the properties of the 2 strains of the variola virus which had undergone 50 passages in cell cultures of human and bovine embryo skin.

Findings

In analyzing the literary data and the results of our own investigations, a conclusion can be made that at the present time sufficient proof has not been obtained testifying to the possibility of the experimental transformation of the variola virus into vaccinia virus. However, does this say that the vaccinia and variola viruses have a different origin and exist in nature independently from each other? We do not consider this so. The presence in different species of animals of variolous infection with different clinical symptoms and caused by viruses which are different from each other in one or the other property, but which always have a certain degree of morphological and antigenic affinity between themselves [3, 6, 7, 15], testifies exactly against this. Apparently the opinion of Bernet /1/ holds true. This is that the viruses of human smallpox and related diseases of mammals have the same common predecessor which subsequently subdivided into various types with host-organisms indigenous to each individual type. The vegetation in the cells of animals of various species guaranteed to each of them specifically individual pathogenic properties. And though at the present time it is impossible to say exactly which mammal was the initial host of the variolous virus-ancestor, it can hardly be doubted that the existence of many varieties of related variolous viruses in nature is the result of a thousand years of evolution, connected with the processes of adaptation variability and selection.

Conclusions

1. The two strains of variola major virus multiplied well during the process of 50 successive passages in cell cultures of human and bovine embryo skin, exerting a cytopathological effect on cells and accumulating in the cultural fluid in titers from 10^{-3} up to 10^{-5} TCD_{50}/0.1 ml.

2. Changes in the properties of the variola virus did not take place as a result of lengthy cultivation under the stated conditions: Similar to the initial condition, both viral strains remained non-cytopathological for cell cultures of chick embryos and in the chorio-allantois of developing chick embryos and in a HEp-1 cell culture caused morphological changes which were sharply different from those caused by the cowpox and vaccinia viruses.

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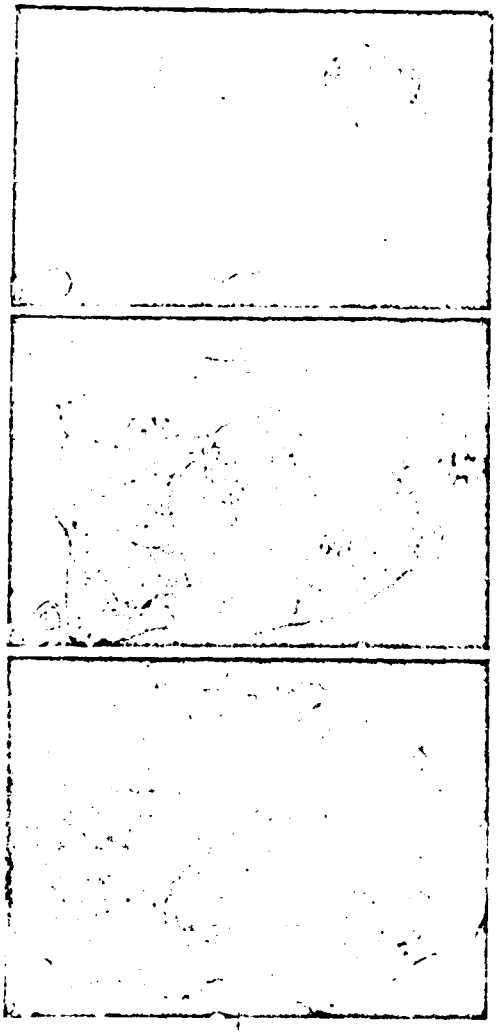
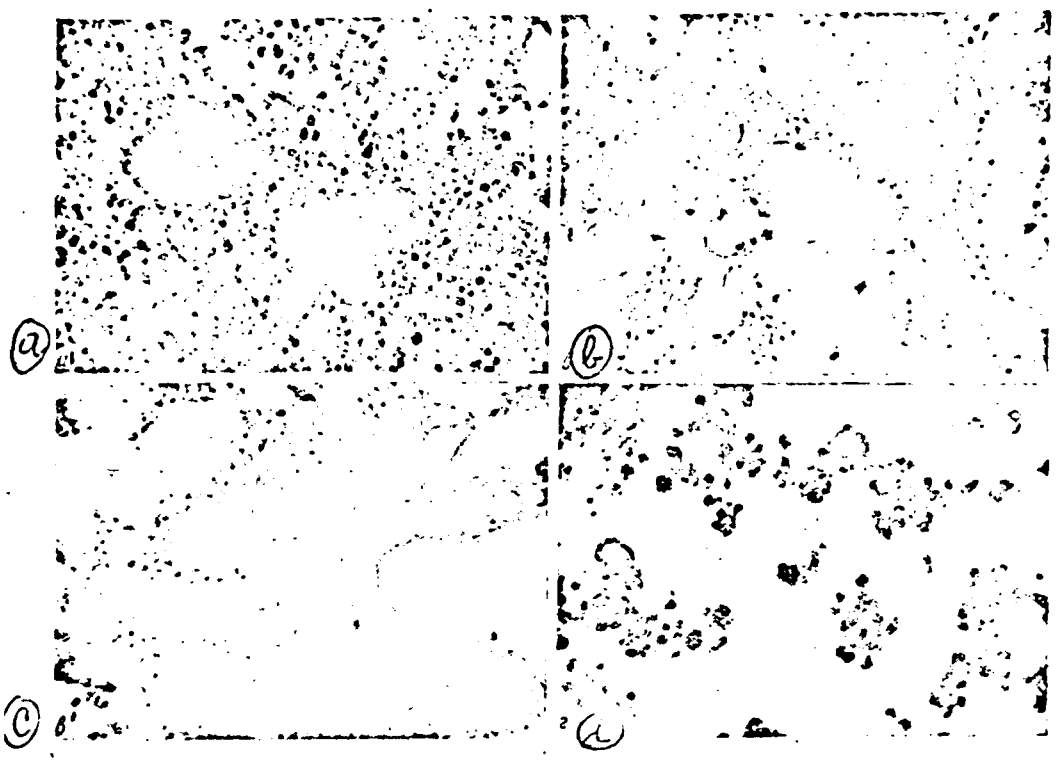


Figure 1. View of the chorio-allantoic membrane of a chick embryo, infected with the virus of variola (a), cowpox (b) and ovovaccinia (c). X2.



Culture of HEp-1 cells, infected with the virus of variola (a), cowpox (b), ovovaccinia (c), and dermovaccine (d). Romanovskiy--Giemsa stain. X150.

Characteristics of the initial properties of the strains of variola, vaccinia and cowpox viruses studied

Virus	Source and method of cultivation up until present investigation	Chick embryos		HEp-1 cell culture		Cell culture of chick embryos		Titer of infecting ability on rabbits	Titer of hemagglutination
		Titer of infecting ability	Nature of lesions on chorio-allantois	Titer of infecting ability	Nature of cyto-pathological effect	Titer of infecting ability	Nature of cytopatho-logical effect		
Variola	Strain No 1, isolated from smallpox patient (scab) in developing chick embryos, after 10 passages	10^{-6}	Minute, sharply outlined, w/ dense raised center, surrounded by hyaline peripheral zone (fig1,a)	10^{-6}	Focal lesions w/ symptoms of proliferation (fig.2,a)	0	Absent	$10^{-4}/51$	1/320
	Strain No 2, isolated from scab of smallpox patient in cell culture of human embryo lung, after 2 passages	10^{-6}	Same	10^{-6}	Same	0	"	$10^{-3.5}/45$	1/2
Cowpox	Strain No 1, isolated from scabs of cow w/cowpox in developing chick embryos after one passage	10^{-7}	Various sizes, w/ clear center & thickened periphery, sometimes mixed (fig.1,b)	10^{-7}	Multinuclear vacuolized symplasts (fig,2,b)	10^{-7}	Degeneration and lysis of cells	$10^{-5}/78$	$\frac{1}{1280}$
	Strain No 1, obtained from Krasnodar San.-Epidem.Station & after 531 passages in developing chick embryos	10^{-8}	Diffuse, with hyaline periphery and more dense, sometimes ulcerous center (fig. 1,C)	10^{-8}	Same (fig.2,c)	10^{-8}	Same	$10^{-6}/99$	$\frac{1}{2560}$

Continuation of table

Virus	Source and method of cultivation up until present investigation	Chick embryos		HEp-1 cell culture		Cell culture of chick embryos		Titer of infecting ability on rabbits	Titer of homogenization
		Titer of infecting ability	Nature of lesions on chorio-allantois	Titer of infecting ability	Nature of cyto-pathological effect	Titer of infecting ability	Nature of cyto-pathological effect		
Vaccinia	Strain No 2, Institute of Epi. & Micro./Gamaleya; passages - bull-ass-bull	10^{-7}	Diffuse with hyaline periphery and more dense, often ulcerous center(fig.1,c)	10^{-7}	Disruption of cellular bonds & breaking up of cultures into sectors of grouped cells (fig.2, d)	10^{-6}	Degeneration and lysis of cells	$10^{-4.5}/75$	1/3
	Strain No 3 from Odessa Ins.Vaccines & Sera; passages bull-sheep-rabbit -bull	10^{-6}	Same	10^{-6}	Same	10^{-6}	Same	$10^{-4}/69$	1/4
	Strain No4 from Minsk Ins.Epi. & Micro.; passages bull--bull	10^{-6}	Same	10^{-6}	Same	10^{-6}	Same	$10^{-4}/64$	1/4